Effects of chain length of saturated fatty acids on Aβ generation in SH-SY5Y cells

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Abstract:

Many studies have shown that saturated fat diet increases the risk of AD. Recently saturated very long chain fatty acids (VLCFAs) have been found be accumulated in AD patients. The variety of saturated fatty acids are found in the diets and human bodies. However, it is not clear which one or more fatty acids are involved in AD pathogenesis. This study investigated the effects of three saturated fatty acids with different carbon chain length (C16:0, C20:0, and C26:0) on amyloid precursor protein (APP) processing and amyloid-β peptide (Aβ) generation. Here, SH-SY5Y cells were treated with vehicle, C16:0, C20:0, and C26:0 (10 μmol/L, 24 h). Compared to the vehicle, C16:0 did not cause any significantly change in APP processing and Aβ generation. C20:0 and C26:0 increased Aβ levels and the expressions of APP, β- and γ-secretase and decreased the expression of α-secretase, and C26:0 had the strongest effects among three fatty acids. Moreover, C20:0 and C26:0 significantly increased reactive oxygen species (ROS), and C16:0 had no such effect. These data indicate that saturated fatty acids with different carbon chain length (C16:0, C20:0 and C26:0) have different effects on the process of Aβ generation, and fatty acids with longer chain (C20:0 and C26:0) have more potential to promote Aβ production and an underlying mechanism of fatty acids action may be related to the elevated oxidative stress. This work supports saturated very long chain fatty acids may play a potential role in the pathogenesis of AD.

Keywords: Saturated fatty acid | Very long chain fatty acids | Fatty acid chain length | Amyloid-B | Alzheimer's disease | Reactive oxygen species

Article:

1. Introduction

Alzheimer's disease (AD) is a serious neurodegenerative disease with progressive cognitive and memory impairment. Amyloid-β (Aβ) plaque is one of the pathological hallmarks in AD brain. Aβ is typically composed of 36–43 amino acids that are derived from amyloid precursor protein (APP) through sequential cleavage by β-site APP-cleaving enzyme 1 (BACE1) and γ-

secretase complex. Conversely, APP can be cleaved by the major α-secretases, ADAM10, at α-site within Aβ sequence preventing Aβ formation [20]. Studies have shown that the excess of Aβ can cause a cascade of pathogenic events such as amyloid deposition, abnormal phosphorylation of tau protein, neurofibrillary tangles, and eventually lead to synaptic dysfunction and typical pathological changes in AD [17]. However, it is still unclear so far which factor triggers the Aβ elevation in AD pathogenesis.

Many studies have shown that saturated fat diet increases the risk of AD [2]. and fatty acid profiles are altered in AD brain [4,5]. Recently, the relationship between saturated very long chain fatty acids (VLCFAs) and AD is increasingly concerned by researchers. Studies have found VLCFAs (mainly C22:0, C24:0 and C26:0) are increased both in AD brain and blood [11,13,22]. VLCFAs have also been shown to damage cell growth and mitochondrial functions of human neuronal SK-NB-E cells [23]. Our pervious study founded $A\beta_{40}$ levels were positively correlated with C24:0 and C26:0 levels in the brain cortex of rats treated with thioridazine [18]. Moreover, an in vitro study reported that an increase in the ratio of C20:0 to C18:0 in GD1b-ganglioside enhanced Aβ assembly [15]. These studies suggest that saturated fatty acids with different chain lengths could play different roles in the pathogenesis of AD. Therefore, we tempted to speculate that saturated fatty acids with different chain lengths could have different effects on Aβ generation.

In the present study, we elucidated the effect of three saturated fatty acids with different chain lengths (*i.e.*, C16:0, C20:0 and C26:0) on the APP processing enzymes and Aβ production. We used SH-SY5Y human neuroblastoma cell as a cell model, screened the suitable concentration of these fatty acids and determined Aβ concentration and the protein expression levels of APP, ADAM10, BACE1 and Presenilin 1 (PS1, the catalytic core of γ-secretase). These data provide evidence for the effects of fatty acid chain lengths on APP processing and Aβ production.

2. Materials and methods

2.1. Cell culture and treatments

SH-SY5Y cells (ATCC: CRL-2266) were cultured in DMEM/F12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA). Palmitic acid (C16:0), arachidic acid (C20:0) and hexacosanoic acid (C26:0) (Sigma-Aldrich, USA) were solubilized in α-cyclodextrin (Sigma-Aldrich, USA) as previously described [8]. MTT and LDH assays were used to assess cell viability and cytotoxicity for screening the optimal concentration of fatty acids. The concentration (10 μmol/L) of three fatty acids without cytotoxicity was chosen as the optimal concentration. For fatty acid treatments, SH-SY5Y cells were incubated in fresh medium containing DMEM/F12 with 1% BSA (Equitech-Bio, USA) and a final concentration of fatty acids (C16:0, C20:0, or C26:0) in 10 μmol/L was used to treat the cells for 24 h. The corresponding vehicle was similarly diluted to be used as the control.

2.2. Assessing the cell viability by MTT assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells/100 μL. After incubation in normal DMEM/F12 containing 10% FBS for 24 h, cells were treated for another 24 h with

C16:0, C20:0, or C26:0 at different concentrations (1.0, 1.5, 3.0, 6.0, 12, 24 and 48 μmol/L) in DMEM/F12 with 1% BSA. Then cells were incubated with 0.5 mg/ml MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) at 37 ℃ for 4 h. The colored formazan crystal produced from the MTT was dissolved in 100 μl of dimethyl sulfoxide. The optical density values of the solutions were measured at 570 nm using a plate reader (BioRad, USA).

2.3. Detecting the integrity of cell membranes by LDH assay

Lactate dehydrogenase (LDH) assay kit (Beyotime, China) was used to detect the integrity of the cell membrane. After SH-SY5Y cells were exposed to C16:0, C20:0 or C26:0 (10 μmol/L) for 24 h, LDH released in the medium was measured. The reduction of nicotinamide adenine dinucleotide (NAD) by the presence of LDH results in the formation of a tetrazolium dye. The optical density values of the solutions were measured at 490 nm using a plate reader (BioRad, USA).

2.4. Quantification of secreted $A\beta_{40}$ by ELISA

After cells were treated with fatty acids for 24 h, cell culture supernatants were harvested for Aβ measurement with human $A\beta_{40}$ ELISA kit (Roche, Germany). Intact protease inhibitor mixture was added to the supernatant to prevent Aβ degradation and cell debris was removed by centrifugation at 3,000 *g* for 10 min at 4 ℃. The values were read at 450 nm using plate reader (BioRad, USA). $A\beta_{40}$ concentrations were determined by comparing the values of the samples to the standard curve. Each sample was analyzed in triplicate, and sample values were expressed as pg/mL.

2.5. Protein analysis by Western blot

For Western blot analyses, cells were harvested and lysed on ice in RIPA buffer containing 50 mM Tris-HCl, pH 6.8, 5% -mercaptoethanol, 2% SDS, and protease inhibitors. The lysate was collected and centrifuged at 12,000*g* 4 ℃ for 20 min. Protein concentration was determined by the BCA assay (Beyotime, China). Equal amounts of protein (20 μg) were loaded and separated by SDS-PAGE. Antibodies and their dilutions used in this study include anti-APP (1:1000, A8717, Sigma, USA), anti-BACE1 (1:1000, Proteintech, USA), anti-ADAM10 (1:1000, Cell Signaling Technology, USA), anti-PS1 (1:1000, Cell Signaling Technology, USA) and anti-β-actin (1:5000, Shanghai Haro Bio, China) as an internal reference control.

2.6. Determination of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) was determined by the ROS assay kit (Beyotime, China). After the cells were treated with fatty acids for 24 h, culture supernatants were removed. Cells were incubated with 10 μmol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA) at 37 ℃ for 20 min in the dark. After washing with serum-free medium, cells were harvested for further analysis using the fluorescence spectrophotometer (Thermo-Fisher, USA). Fluorescence is monitored at excitation wavelength 488 nm and emission wavelength 525 nm.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical significance between control and experimental groups using SPSS17.0 software (SPSS Inc, Chicago, IL) for Windows. P value less than 0.05 was considered as statistical significance.

3. Results

3.1. Screening the optimal fatty acid concentration

Some studies have shown that higher concentrations of fatty acids have cytotoxicity which was related to the carbon chain length and the number of double bonds [12]. To screen the optimal fatty acid concentration, we used MTT to assay cell viability and demonstrated that three fatty acids (C16:0, C20:0 and C26:0) impaired cell viability in a dose-dependent manner after treatment of 24 h (Fig. 1). From the figure, we concluded 12 μmol/L is the highest concentration of three fatty acids that are non-toxic to the cells. Furthermore, we used the LDH assay showing that none of these compounds have found cytotoxicity at the concentration of 10 μmol/L (Fig. 2). Thus 10 μmol/L of C16:0, C20:0 and C26:0 was chosen as the optimal concentration for the following experiments.

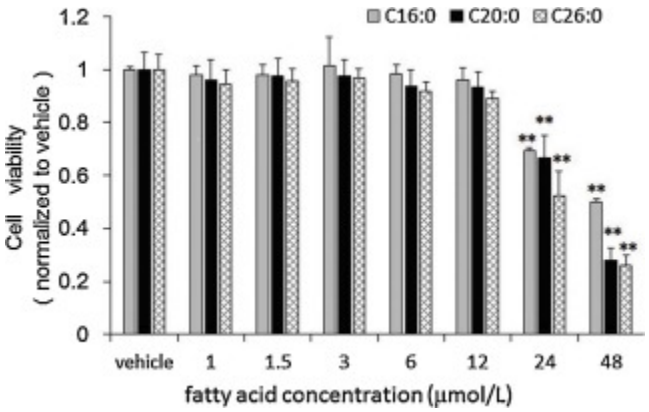

Fig. 1. Cell viability of SH-SY5Y cells detected by MTT assay. Cells were treated with C16:0, C20:0 and C26:0 at different concentrations for 24 h, then MTT assay was carried out to assess cell viability. $n = 3$ in each group; $^{**}p < 0.01$, *vs* vehicle control.

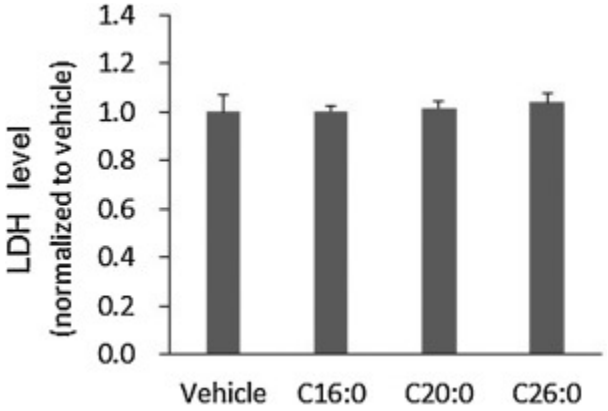

Fig. 2. LDH level in culture medium of SH-SY5Y cells. Cells were exposed with fatty acids at 10 μmol/L for 24 h, then cell culture supernatants were used to determine LDH. $n = 6$ in each group.

3.2. The levels of $A\beta_{40}$ in culture media

As shown in Fig. 3, compared with the vehicle control, $A\beta_{40}$ level in C26:0 group was significantly increased by 1.2-folds ($P < 0.05$). Moreover, $A\beta_{40}$ had a slight increase in C20:0 group compared with the vehicle ($P = 0.11$), although there was no significant difference ($P > 0.05$). There were no significant differences between the other groups ($P > 0.05$).

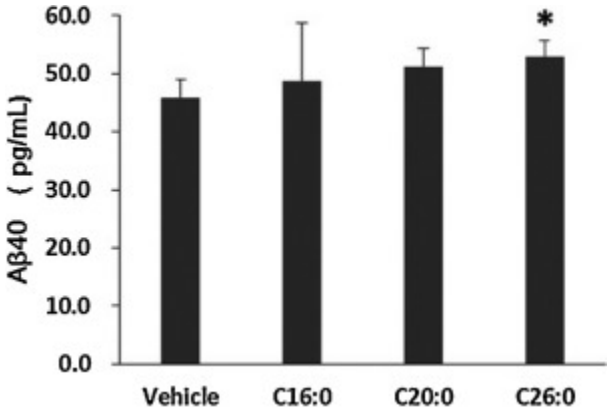

Fig. 3. Aβ40 level in culture medium of SH-SY5Y cells detected by ELISA. After cells were exposed with fatty acids at 10 μmol/L for 24 h, cell culture supernatants were harvested for Aβ measurement with $A\beta_{40}$ ELISA kit. $n = 6$ in each group; $^{*}p < 0.05$, *vs* vehicle control.

3.3. The protein levels of APP, BACE1, PS1, and ADAM10

As shown in Fig. 4, compared with the vehicle group, APP protein levels in C20:0 and C26:0 group were increased by 1.5 and 2.1 folds, respectively ($P = 0.056$, $P < 0.01$). APP level in C26:0 group was significantly higher than the C16:0 and C20:0 group, respectively ($P < 0.01$, $P < 0.05$).

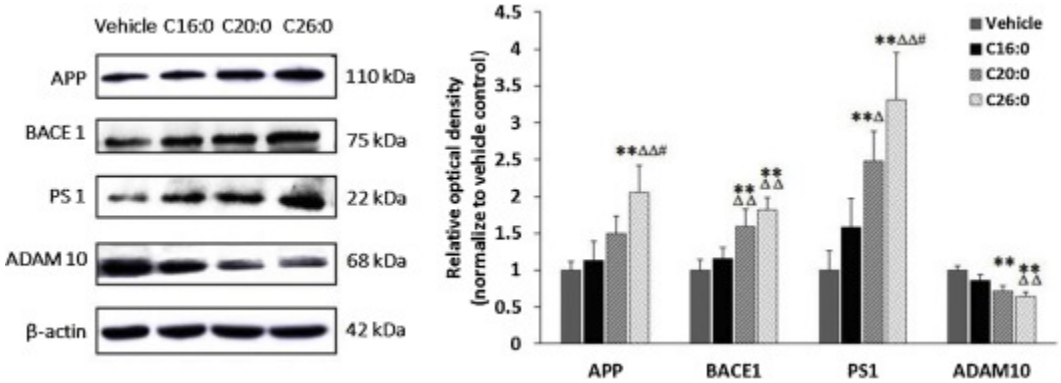

Fig. 4. The relative protein expressions of APP, BACE1, PS1 and ADAM10 quantified by Western bolt. After treatment with fatty acids at 10 μmol/L for 24 h, cell lysates were prepared. APP, BACE1, PS, and ADAM10 in cell lysates were analyzed by Western blot and β-actin was used as internal control. $n = 4$ in each group; $^{**}p < 0.01$, *vs* vehicle control; $^{\Delta}p < 0.05$ *vs* C16:0 group; $^{\Delta\Delta}p < 0.01$ *vs* C16:0 group; $^{\#}p < 0.05$ *vs* C20:0 group.

Subsequently, our results showed that BACE1 protein level was obviously increased in C20:0 and C26:0 group by 1.6 and 1.8 folds respectively ($P < 0.01$ in both groups), compared with the

vehicle group (Fig. 4). BACE1 level in C20:0 and C26:0 group were also higher than C16:0 group ($P < 0.01$ in both groups).

Similarly, PS1 protein level in C20:0 and C26:0 group were remarkably increased by 2.5 and 3.3 folds compared with the vehicle group ($P < 0.01$ in both groups) (Fig. 4). Furthermore, PS1 level in C20:0 and C26:0 group were higher than C16:0 group ($P < 0.05$, $P < 0.01$), and PS1 level in C26:0 group was also higher than C20:0 group ($P < 0.05$) (Fig. 4).

By contrast, the expression of ADAM10 was found to significant decrease by 29% and 36% ($P < 0.01$ in both groups) in C20:0 and C26:0 group, compared with the vehicle group (Fig. 4). Moreover, ADAM10 protein level in C26:0 group showed a 22% reduction compared with the C16:0 group ($P < 0.01$).

3.4. The level of intracellular ROS

Oxidative stress is known to play an important role in the pathogenesis of AD. To investigate whether oxidative stress is involved in the fatty acid-induced increase of Aβ production, we detected the level of intracellular ROS. As shown in Fig. 5, there was no meaningful difference in ROS level between C16:0 group and vehicle group ($P > 0.05$). However, the levels of ROS in C20:0 and C26:0 were significantly higher than that in the vehicle and C16:0 group ($P < 0.01$, all). ROS level in C26:0 was also considerably higher than that in C20:0 ($P < 0.01$) (Fig. 5).

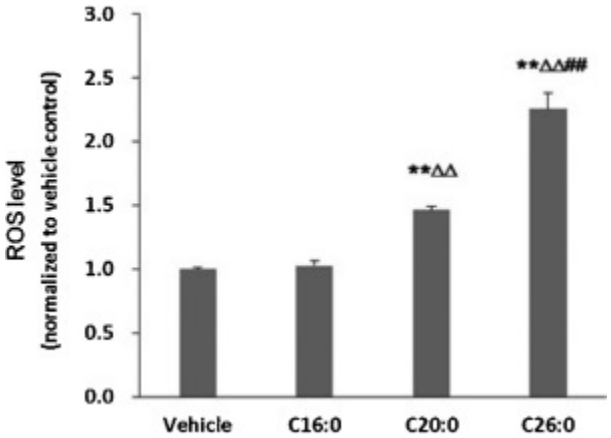

Fig. 5. Intracellular ROS level of SH-SY5Y cells. After the cells were treated with fatty acids at 10 μmol/L for 24 h, cells were used to measure ROS using 2′, 7′-dichlorofluorescein diacetate. $n = 6$ in each group; $^{**}p < 0.01$, *vs* vehicle control; $^{\Delta\Delta}p < 0.01$ *vs* C16:0 group; $^{\#\#}p < 0.01$, *vs* C20:0 group.

4. Discussion

Studies have shown that higher concentrations of fatty acids have cytotoxicity [12]. In line with these findings, using MTT assay, we found that C16:0, C20:0 and C26:0 had toxicity to cells when the concentrations exceeded 12 μmol/L. Considering that the experimental concentrations should maintain the normal viability and basic function of cells, we further used LDH assay to confirm that the concentration of 10 μmol/L is the optimal concentration that has not observed toxic effects.

In this study, under the present experimental conditions, we found fatty acids (C16:0, C20:0 and C26:0) with different chain length had different effects on the process of Aβ generation. Both C20:0 and C26:0 increased the levels of APP, BACE1 and PS1, decreased ADAM10 level and increased Aβ generation. C26:0 had stronger effects on all the testing items than C20:0. But C16:0, with a relatively short chain, had little effect on the process of Aβ generation. These results suggest that fatty acids with longer carbon chain length are more likely to increase APP amyloidogenic processing and Aβ level. Recently it was reported that C16:0-BSA treatment (50 μmol/L) increased the expressions of APP and BACE1, and Aβ production in SK-N-MC cells [10]. However, our experimental results were inconsistent with this study's results. We suspect the reason could be that the different concentrations of C16:0 were used in the present experiment with the previous study.

Fatty acids are important components of the phospholipids that are involved in biofilm formation. Biological membrane properties and cellular functions could be altered by being incorporated different types of fatty acids [7,21]. All APP-cleaving secretases, as well as APP, are transmembrane proteins, and the proteolytic processing of APP are taken place in its transmembrane domain [6]. An in vitro experiment has shown membrane lipids have greatly influence on γ-secretase activity and processivity, and the increase in fatty acid carbon chain length [[12], [13], [14]] increased γ-activity and reduced the $A\beta_{42}/A\beta_{40}$ ratio [9]. Another in vitro study reported that an increase in the ratio of C20:0 to C18:0 in GD1b-ganglioside enhanced Aβ assembly [15]. Our results further demonstrate that fatty acids with different carbon chain length have important effects on APP proteolytic processing and Aβ generation. The possible underlying mechanism of their actions might be due to the changed membrane properties by the different fatty acids.

Oxidative stress is known as one of the earliest causing events in the early stages of AD and plays an important role in the pathogenesis and development of AD [14]. Oxidative stress promotes Aβ production in the neurons and increasing Aβ can further increase oxidative stress [3]. Saturated fatty acids were previously reported to induce oxidative stress [16]. There are also evidences that VLCFA accumulation stimulated oxidative stress leading to lipid peroxidation, decreased the antioxidant defenses and caused overproduction of reactive oxygen species and nitrogen species [1,19,23]. This study also showed that both C20:0 and C26:0 increased intracellular ROS significantly. Based on the oxidative stress theory of AD pathogenesis and present results, we consider that oxidative stress may be implicated in the mechanism of fatty acid-induced increase of Aβ, APP, BACE1, and PS1.

A large scale of evidence from epidemiological and animal studies have shown that saturated fat increases the risk of AD [2]. The variety of saturated fatty acids are found in the diets. However, it is not clear which one or more fatty acids are involved in AD pathogenesis. Recently saturated VLCFAs have been shown to accumulate in AD patients and damage cell growth and mitochondrial functions [11,13,22,23]. Some reports also have demonstrated that Aβ level was positively correlated with C26:0 or C24:0 [19]. Our study provides further evidence to support that VLCFAs may possibly play an important role in AD pathogenesis.

In summary, our data indicate that saturated fatty acids with different carbon chain length (C16:0, C20:0 and C26:0) have different effects on the process of Aβ generation, and fatty acids

with longer chain (C20:0 and C26:0) have more potential to promote Aβ production and an underlying mechanism of fatty acids action may be related to the elevated oxidative stress. This work supports saturated very long chain fatty acids may play a potential role in the pathogenesis of AD.

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